Live Conjunctiva-Associated Lymphoid Tissue Analysis in Rabbit under Inflammatory Stimuli Using In Vivo Confocal Microscopy

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PURPOSE. Conjunctiva-associated lymphoid tissue (CALT) plays an important role in ocular surface immunity. No study until now has been able to show its in vivo aspects, and few have demonstrated its reactions after pathologic patterns. The authors investigated in rabbit eyes the cell reactions occurring in the organized CALT during conjunctival inflammation models.

METHODS. Using in vivo confocal microscopy (IVCM), the authors analyzed, for the first time in vivo inflammatory cell infiltration and circulation inside the lymph vessels in rabbit CALT after inflammatory stimuli (LPS, TNFα, LPS+anti-TNF). Cresyl violet staining was performed to observe the morphology of CALT, and immunohistochemistry was performed in whole mount conjunctiva and cryosections for detecting CD45+ lymphocytes. Human CALT in vivo aspects were also explored in a patient with vernal keratoconjunctivitis (VKC).

RESULTS. The conjunctivitis model induced by LPS was characterized by inflammatory cell infiltration in the dome and intrafollicular layers of CALT and cell circulation inside the lymph vessels. TNF alone induced moderate inflammatory infiltration in CALT. However anti-TNF antibodies could significantly decrease LPS/TNFα-induced inflammation. CD45+ lymphocytes were strongly expressed in the CALT after injection of LPS or TNFα at 4 hours and decreased with injection of anti-TNFα. The authors also showed the presence of the CALT pattern in VKC.

CONCLUSIONS. The authors showed for the first time the in vivo aspects of normal and pathologic cell reactions in rabbit CALT after inflammatory stimuli. IVCM-CALT could be a pertinent tool in the future for the comprehension of ocular surface defense mechanisms, and inflammatory cell analysis in CALT could constitute a new criterion for evaluating ocular surface immunity.

The eye is constantly exposed to many environmental stimuli and aggressions such as desiccation, topical drugs, allergens, pollutants, bacteria, and viruses. The ocular surface, especially the conjunctiva, necessitates this permanent stimulation and lymphocyte trafficking for physiological protection against xenobiotics. To better describe the role of the resident mucosal immune system of the ocular surface, the concept of eye-associated lymphoid tissue (EALT) was proposed and termed as a new component, including conjunctiva-associated lymphoid tissue (CALT) and lachrymal drainage-associated lymphoid tissue.1 This EALT system was reported to detect antigens and distribute protective factors in the ocular surface.1,2 The search for and identification of CALT-associated structures has progressively taken an important place in the understanding of innate and acquired ocular surface immunity. According to reports in the literature, debate continues regarding the existence of CALT and its functions. Knop et al.1–3 confirmed its existence as an autonomous lymphoid organization and its important role in ocular surface immunity and defense. The lymphoid cells of CALT are involved in diverse functions, such as antimicrobial defense, hypersensitivity (allergy), allograft rejection and immune tolerance.

CALT is organized both diffusely and in aggregates or follicles (principally the lymphocytes) in the lamina propria.4 In postmortem human eyes, follicles were mainly observed in the tarsal and orbital conjunctiva of the upper and lower lids, with fewer in the fornix and rare follicles in the bulbar conjunctiva. Immunohistochemistry confirmed that the follicles were accumulations of CD20+ B cells diffusely associated to CD3+ T cells in the periphery. In addition to T and B lymphocytes, macrophages, plasma cells, and dendritic cells (DCs) were found.5 In the parafollicular area of the CALT structure can be found specialized vessels with tall endothelia, called high endothelial venules (HEVs), that are important for lymphocyte homing to the conjunctiva.6 In contrast to mice and rats, rabbits are a good model in which to study CALT and the specialized follicle-associated epithelium (FAE). Rabbits have not only an organized CALT (O-CALT) but also functional M cells able to subject lectins, latex beads, and bacteria in the overlying FAE to transcytosis. In addition, age-related changes in rabbit O-CALT are similar to what has been reported in human conjunctiva.7

Previous studies that described normal rabbit CALT and its function have been reported, but few have been conducted on its reactions after inflammatory stimuli. Moreover, to our knowledge, to date no study has been able to show in vivo the normal and stimulated aspects of CALT. For this reason, we conducted an in vivo investigation of the rabbit O-CALT follicles using corneal in vivo confocal microscopy (IVCM) after various inflammatory stimuli, such as lipopolysaccharide (LPS) and tumor necrosis factor-alpha (TNF). We chose LPS as a major inflammatory stimulus because LPS, through the action

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of TNF, plays important roles in lymphocyte activation, proliferation, and migration.\textsuperscript{8,9}

In previous studies, we showed the value of IVCM in ocular surface evaluation in different normal and pathologic conditions using various inflammatory or toxic challenges.\textsuperscript{10,11} A scoring system was constructed to quantify and standardize the IVCM patterns for toxicologic and immunologic studies.\textsuperscript{10,11} In this study, we focused our investigations on identifying O-CALT structure and changes in vivo. CALT has never been observed in vivo, even with the use of IVCM techniques, because of its peripheral location under the eyelid edge, an area in which the hyperreflectivity of the substantia propria and the surrounding conjunctiva make identifying such complex structures difficult. In contrast to previous reports from ex vivo or even postmortem investigations, we succeeded in identifying in vivo rabbit O-CALT in normal, inflammatory, and toxic conditions and compared it to exceptional and original IVCM CALT images taken for the first time in humans, based on the patterns found in rabbits. CALT activation could, therefore, become a new important criterion for evaluating ocular surface inflammation and immunity.

**METHODS**

**Animals**

All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male New Zealand Albino rabbits (age, 2.5 months; weight, 2 kg) were used. Before all experiments, ocular surface integrity was examined by slit-lamp microscopy, and the CALT aspect was examined using IVCM. A mixture of ketamine (35 mg/kg; Imalgene 500; Merial, Lyon, France) and xylazine (5 mg/kg; Bayer, Puteaux, France) was used to anesthetize the animals by intramuscular injections. In total, 30 rabbits were used for the conjunctivitis model.

**Subconjunctival Injection of PBS and LPS/TNF with or without Anti-TNF**

A 29-gauge insulin syringe (UltiFine; Ulti Med, St. Paul, MN) was used for subconjunctival injections. A single 10-μL injection of sterile phosphate-buffered saline (PBS), LPS from *Escherichia coli* (Sigma Aldrich, St. Louis, MO) at 200 ng/μL (2 μg/injection),\textsuperscript{12} or TNF at 200 ng/μL (2 μg/injection; R&D Systems, Lille, France) was administered to the superior bulbar conjunctiva of each eye of each rabbit. In two other groups, 5 μL LPS or 400 ng/μL TNF and 5 μL neutralizing TNF (MAb210; R&D Systems, Lille, France) at 20 ng/μL was coinjected as in a previous study.\textsuperscript{12}

IVCM was conducted over 24 hours in five groups (PBS, LPS, TNF, LPS plus anti-TNF, or TNF plus anti-TNF) of six rabbits each: three rabbits at 4 hours and three rabbits at 24 hours. At selected time points, one rabbit from each group was killed for the dissection of whole-mount conjunctiva, and two other rabbits were killed for the preparations of cryosections. The number of rabbits used is shown in Table 1.

**In Vivo Confocal Microscopy Observation**

The laser scanning IVCM Heidelberg Retina Tomograph (HRT) II/Rostock Cornea Module (RCM; Heidelberg Engineering GmbH, Heidelberg, Germany) was used.\textsuperscript{10,12} No special dye injection or other special preparation was needed. For the observation of the CALT structure, the superior conjunctiva was turned slightly, as shown in Figure 1A. By delicately moving the objective of the IVCM over the entire conjunctiva and partial fornix, the lymphoid follicles of CALT could be more clearly observed in the upper tarsal conjunctiva. The depth of CALT was checked manually by the x-y position of the optical section; the focus position (μm) was automatically calculated by the HRT II/RCM. For all eyes, at least three to five visible follicles were recorded and analyzed by IVCM. IVCM analysis in the conjunctiva was more difficult than in the cornea, which was transparent and could be observed to the corneal endothelium (approximately 400 μm). In the conjunctiva, the IVCM could detect from 0 μm to 80 to 90. It could not detect the entire depth of the conjunctiva because of the high hyperreflectivity of underlying tissues, such as sclera for bulbar conjunctiva. CALT follicles could not be clearly visualized over 70 to 80 μm. Nevertheless, the follicle diameters in the x-y axis could be more accurately analyzed with the 400 × 400 μm IVCM scale. To standardize the IVCM analysis of CALT, the zone from 0 to 15 μm was defined as the superficial epithelial dome, and the layers from 15 to 30 μm were defined as the intrafollicular layers. Infiltrating inflammatory cells were counted using a cell counting program. The final counts of inflammatory cells (lymphocytes, polymorphonuclear cells, and dendritiform cells) were the averages of the five follicles of two or three rabbits.

To demonstrate the specificity of the IVCM CALT observation, the CALT structure, normal cervical lymph node (CLNs), submandibular glands, lachrymal glands, and meibomian glands were also dissected after animal kill and compared.

Conversely, the IVCM has been routinely used for several years in our clinical ophthalmology practice to detect the ocular surface anomalies in patients, particularly in those with chronic or recurrent diseases, such as dry eye and allergy.\textsuperscript{13–15} Until this experiment, CALT had not been recognized in any of these images. After our rabbit findings, we undertook a systematic assessment of IVCM images obtained in the conjunctiva of patients with severe inflammatory diseases. We selected suggestive patterns and compared them to the rabbit images.

**CALT Immunohistology in Whole-Mount Conjunctiva and Cryosections**

At 4 hours, rabbits were killed by overdose injection of pentobarbital. The whole-mount conjunctivae of the killed rabbits were fixed in 4% paraformaldehyde (PFA) for 4 hours at 4°C and were incubated with mouse anti-rabbit CD45 (1:50; BCL1142; Cymbus Biotechnology, Chandlers Ford, UK) for 4 hours at 4°C and then with anti-mouse immunoglobulin Alexa Fluor 488 (1:500) for 1 hour. Propidium iodide was used to stain the nuclei. An in vitro confocal microscope (PCM2000; Nikon, Tokyo, Japan) was used to observe the entirely dissected nontransparent conjunctiva. For the preparation of cryosections, excisedue eyes were fixed in 4% PFA for 4 hours at 4°C, and the 10-μm cryosections were prepared and incubated with antibodies directed against rabbit CD45 (1:50; Cymbus Biotechnology) for 2 hours at 4°C. Sections were then stained with secondary antibody and later with propidium iodide. Images were digitized with a fluorescence microscope (BX-UCB; Olympus, Melville, NY).

Immunopositive cells were counted on whole-mount or cryosections from three rabbits per treatment using a 100 × 100-μm reticulum. The centers of CALT follicles were counted, and three sections were used for each treatment. Results were presented as means ± SD for each treatment.

**Cresyl Violet Staining**

Other cryosections were washed in distilled water, dehydrated into ethanol, and stained by cresyl violet solution (1%, no. 5235; Merck, Fontenay-sous-Bois, France) for 30 minutes. The samples were then air-dried and mounted in medium (Eukitt; CML, Nemours, France).
Statistical Analysis

Results were expressed as means ± SE. Groups for analysis were compared using factorial analysis of variance (ANOVA) followed by the Fisher method (Statview V; SAS Institute Inc., Cary, NC).

RESULTS

IVCM Images of Cervical Lymph Nodes and CALT

As observed using IVCM (Fig. 1A), CALT appeared as a round or oval structure and was darker than the surrounding white and hyperreflective conjunctival substantia propria. In the dome, the follicle could be seen (Fig. 1B) bulging slightly from the surrounding conjunctival epithelium. In the intrafollicular layers (Fig. 1C), we distinguished a network of fibrils containing numerous small, hyperreflective gray patterns (arrows), which might have been lymphocytes. Follicles of the rabbit CALT measured from 400 μm (Fig. 1B, C) to 1100 μm (Fig. 1D), consistent with postmortem human eye patterns16; Figure 1D combines four photographs of the same follicle at the same depth. The CALT follicles were observed especially on the tarsal or orbital conjunctival areas with rare elements in the bulbar region, consistent with a previous histologic report.7

To prove the specificity of the IVCM CALT patterns, we compared CALT structures with CLN structures (Fig. 1E), submandibular/lachrymal glands, and meibomian glands of the eyelid (data not shown). We confirmed that the CALT structure was similar to the CLN structure but not to the exocrine gland structure. CLN (Fig. 1E) showed dense masses of cells jammed together in this lymphoid tissue, in which the delicate reticular fibers were invisible. The three-dimensional network of the fibrils inside the CLNs contained numerous small, bright cellular patterns (arrows). However, the hyperreflective patterns visible inside CALT (Figs. 1C, D, arrows) were more difficult to visualize than the CLN patterns, as assessed by IVCM.

In Vivo Images of Rabbit CALT after Subconjunctival Injection of LPS/TNF±Anti-TNF

Compared with normal CALT, 4 hours after injection of PBS, no obvious changes could be observed in the dome (Figs. 2A, U; 13.9 ± 5.0 cells/mm³) and intrafollicular layers (Figs. 2B, V; 0.7 ± 0.7 cells/mm³) of CALT (Movie S1, http://www.iovs.org/cgi/content/full/51/2/1008/DC1); the same tendency was found at day 1 (Figs. 2C [dome], D [intrafollicular layers]).

However, LPS induced substantial inflammatory cell infiltration in the dome (Fig. 2E; 952.8 ± 144.6 cells/mm³) and intrafollicular (Fig. 2F; 99.9 ± 34.9 cells/mm³) layers (P < 0.0001 compared with all other groups in both layers) at hour 4 (Movie S2, http://www.iovs.org/cgi/content/full/51/2/1008/DC1). These inflammatory cells, characterized by their hyperreflectivity, were different from those observed in normal CALT or CALT after the injection of PBS. This infiltration decreased dramatically at 24 hours: the dome returned to a normal aspect, but some inflammatory cells could still be observed in the intrafollicular layers (Figs. 2G [dome, 14.5 ± 7.4 cells/mm³], H [intrafollicular layer, 9.4 ± 4.8 cells/mm³]). At 24 hours, both the activation of lymphatic vessels (arrows) and the inflammatory cell circulation were observed at a relatively high speed after the subconjunctival LPS stimulation.
Compared with LPS, TNF induced less inflammatory cell infiltration in rabbit CALT at 4 hours in the dome (Fig. 2I; 216.2 ± 37.3 cells/mm²) but significantly more than in the control groups (P < 0.01). However, the infiltration mainly occurred in the CALT periphery. We also observed inflammatory cell infiltration in the intrafollicular layer (Fig. 2J; 19.5 ± 8.1 cells/mm²). IVCM showed the in vivo presence of inflammatory cells (arrow) stretching from the CALT periphery toward its center. This inflammatory cell infiltration disappeared at 24 hours (Figs. 2K [dome], L [intrafollicular layer]; fewer than 5 cells/mm² in both areas).

Interestingly, anti-TNF neutralizing antibodies coinjected with LPS inhibited the inflammatory infiltration to a large extent, showing fewer inflammatory patterns in the dome (Fig.
from the mucocyte pattern (Fig. 3E). The small intraepithelial lymphocytes (Fig. 3F, arrow) presented a typical rounded or oval nucleus. In the intrafollicular layer of the CALT follicles, PMNs (i.e., neutrophils and eosinophils) were also observed, as were many macrophage-like cells (Fig. 3G, arrows). After the injection of LPS, eosinophils and macrophages accumulated in large numbers in CALT follicles, which was not observed in the other parts of conjunctiva.

**CD45 Expression in CALT in Whole-Mount Conjunctiva/Cryosections in Conjunctival Challenge Models**

In whole-mount conjunctivas (Fig. 4, line 1) and cryosections (Fig. 4, line 2), we observed CALT follicle structures presenting spherical patterns, protruding from the flat superficial conjunctival layer. These lymphoid follicles were recognized by their slight elevation, an assemblage of cells that was smaller than the epithelial cells, and sometimes the distinctive branching pattern of lymph vessels around the follicle region. Cryosections of the follicles revealed that few or no goblet cells were present within the FAE. At 4 hours, PBS injection induced only a slight expression of CD45 in the follicle area, as recognized by immunohistology in the whole-mount conjunctiva (Fig. 4A) or in the cryosections (Fig. 4B). LPS induced much more abundant and brighter expression of CD45 in the dome of CALT follicles (Figs. 4C, D). The follicle area was densely positive to CD45, especially with small round cells, which were most likely CD45+ lymphocytes. CD45+ cells were found not only in the follicle area but also in the epithelial layer. This CD45 expression decreased at 24 hours after all treatments (data not shown). At 4 hours, TNF also induced an increase of CD45+ inflammatory cells (Figs. 4E, F) that was less intense than that caused by LPS. Coinjection of LPS and anti-TNF antibody induced less CD45+ cell infiltration in the CALT follicle area and around it and in the areas adjacent to the CALT subepithelial zone (Figs. 4G, H).

Superficial CD45+ cells were counted in the whole-mount conjunctiva, and they were also counted in the intrafollicular part of CALT in the cryosections (Fig. 4I). After PBS injection, the CD45+ cells numbered 2066 ± 176 cells/mm² in the whole-mount conjunctiva and 3100 ± 781 cells/mm² in the intrafollicular part of CALT in cryosections. LPS induced the maximal expression of CD45+ cells: 5767 ± 352 cells/mm² in the whole-mount conjunctiva and 10,866 ± 996 cells/mm² in the cryosections (P < 0.006 compared with PBS and P < 0.04 compared with the other three groups). When LPS was coinjected with anti-TNF, the CD45+ cells decreased to 4633 ± 348 cells/mm² in the whole-mount conjunctiva and 3733 ± 392 cells/mm² in cryosections (P < 0.04 compared with LPS injection). The same trend was found when anti-TNF was coinjected with TNF, and the CD45+ cells decreased significantly (1800 ± 115 cells/mm² in whole-mount conjunctiva and 3577 ± 348 cells/mm² in the cryosections) compared with TNF alone (3967 ± 491 cells/mm² in whole-mount conjunctiva and 6166 ± 328 cells/mm² in the cryosections) (P < 0.002). There was no difference between PBS and TNF+anti-TNF injection in CD45+ cell counts.

**Cresyl Violet Staining of PBS- and LPS-Injected CALT**

The conjunctivas of the killed rabbits were prepared as cryosections for cresyl violet staining. After the subconjunctival injection of PBS, we observed only a few scattered lymphocytes or a few lymphoid islets (Fig. 3A) with no other immune cells, such as macrophages or polymorphonuclear cells. However, after the subconjunctival injection of LPS, the CALT follicles increased dramatically in size with darker staining (Fig. 3B). At high magnification, numerous immune cells were found in the epithelial layer of CALT follicles, with polymorphonuclear cells (PMNs; Fig. 3C, arrows), mainly neutrophils and rare eosinophils, characterized by their bilobular nuclei and secretory-specific granules. As described by Knop et al., M cells were identified with typical patterns and folding containing two lymphocytes (Fig. 3D, arrow), which was different...
apparently smaller than, those observed in rabbits. To our knowledge, this is the first in vivo image of mucosa-associated lymphoid tissue in humans (Fig. 5).

**DISCUSSION**

In contrast to the avascular cornea, the other ocular surface-related tissues are vascularized and contain an active lymphatic network participating in antigen presentation and lymphocyte homing. An efficient immune system is mandatory for protecting the extremely fragile intraocular tissues and thus preserving corneal transparency and visual function. Moreover, during eye closure, when blinking or overnight, the structure of CALT overlies the cornea, enhancing cornea immune defense. Therefore, research exploring the conjunctiva-associated immune system has great potential to contribute to a better understanding of ocular surface immunity. The EALT system physiologically plays a protective role, but if it is deregulated it can arouse an inflammatory immune-mediated response and be involved in severe chronic inflammatory ocular surface diseases. Chronic or repeated inflammatory reactions at the ocular surface may lead to a vicious circle of chronic inflammation involving all the ocular surface tissues, with degenerative remodeling and loss of function or dry eye disease. In this

![Image](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932966/ on 07/14/2018)
study, for the first time, we demonstrated in vivo the presence of CALT in rabbit eyes, and its activation after inflammatory stimuli with inflammatory cells visible using a noninvasive technique. Moreover, this dynamic in vivo analysis provides the means to observe the same animal throughout the experiment, before and after the inflammatory stimuli, thus avoiding variations between animals. In addition, the information provided by IVCM, including the topography, size, and cell count of inflammatory cells in CALT structures, accurately reflects the activation status of the whole ocular surface and could become important criteria for evaluating the efficacy of new anti-inflammatory agents.

The results obtained from the conjunctivitis model using LPS, a strong inducer of inflammatory cells in the CALT structure, were consistent with those of previous studies on LPS-induced effects on the ocular surface.12,18 We thus showed that subconjunctival LPS induced severe conjunctival inflammation and apoptosis, which could be inhibited to a large extent by a neutralizing anti-TNF antibody.12 In various corneal injury models, LPS instillation also accentuated the inflammatory and apoptotic effects induced after corneal impairment by means of TNF overexpression.19 In the present study focused on CALT structure, the infiltration of inflammatory cells reached a maximum shortly (4 hours) after the subconjunctival injection of LPS, characterized by numerous CD45+ inflammatory cells, macrophages, and lymphatic vessels. This strong inflammatory cell reaction was significantly inhibited by coinjection of an antibody-neutralizing TNF. Compared with LPS, TNF showed an intrafollicular capacity to induce cellular attraction in CALT, at least as observed by IVCM, with fewer CD45+ cells as assessed by immunohistology.

Normal rats lack O-CALT, but CALT-like lymphoid aggregates have been shown to develop after corneal injury such as corneal transplantation.16 These aggregates contain granulocytes, macrophages, and CD45+/CD8+ T cells in the deeper stroma. In mice repeatedly challenged with topical Chlamydia trachomatis or ovalbumin/cholera toxin, CALT was also detected in the nictitating membrane.20 These CALTs consist mainly of CD45R/B220+ B cells, CD45+/CD8+ T cells, dendritic cells, and macrophages. These studies demonstrate, under pathologic conditions in rodents normally devoid of an obvious CALT structure, that CALT not only appeared and assembled, it also executed its role of stimulating chemotaxis for numerous inflammatory cells.

The lymphocyte circulation occurs in vessels suggestive of HEVs, as identified using IVCM. HEVs could play an important role in the pathogenesis of chronic inflammatory diseases. In a model of experimental autoimmune uveitis, HEV plays a central role in the homing and migration of inflammatory cells into the eye.21,22 The quick inflammatory cell reaction observed in the two models studied herein was also likely attributable to the HEV’s. Complex molecular mechanisms control HEV specialization.23 HEVs expressing lymphocyte homing receptors may play a significant role in lymphocyte homing to the conjunctiva, particularly through vascular adhesion protein-1 and intercellular adhesion molecule-1 as primary addressin/adhesion molecules.6,24,25 Lymphocyte trafficking inside the CALT structure also relies on complex chemokine responses.26 CCR7 plays an important role in guiding DCs to secondary lymphoid nodes during an adaptive immune response.27 The presentation of foreign antigens and the clonal expansion of antigen-specific effector lymphocytes both involve a close collaboration between organized lymphoid tissues and the specialized FAE, including M cells.28

After using IVCM analyses in many animal models of conjunctivitis, keratitis, neovascularization,25 toxicity,10,11,20 and uveitis,30 we succeeded for the first time in visualizing the dynamic changes in lymphoid follicles. IVCM has the major advantage of being noninvasive and allowing follow-up of the disease with no interaction with the animal’s physiological/pathologic responses. Former studies on CALT were restricted to a histologic description, whereas more recent immunohistologic studies investigated only small pieces of tissue obtained from clinical biopsy specimens. A better understanding of in vivo lymphocyte activation could lead to more effective strategies for the treatment and prevention of infectious and autoimmune diseases.31 In humans, studies on CALT were undertaken on conjunctival cul-de-sacs obtained from cadaveric eyes or clinical biopsy specimens with various results, proving that well-organized CALT structures in humans are not constant. CALT follicles were not observed in the newborn, but their number increased before puberty and progressively decreased with age.52 Most of them were secondary oval follicles with an average diameter of 0.3 mm, as also described by Kessing et al.53 Consistent with these studies, our work highlights CALT, for the first time in vivo, in rabbits and also in patients with severe allergic reactions, as the only component of the mucosa-associated lymphoid tissue directly accessible in vivo without surgery or an ex vivo staining procedure. VKC is an allergic ocular surface disease characterized by severe and chronic seasonally antigenic stimulation and inflammation,24 which could explain the visibility of CALT-like structures in these patients. Whether well-organized CALT structures exist in normal eyes in areas that are difficult to access with IVCM or are only visible in highly pathologic conditions remains to be elucidated. Nevertheless, our rabbit study has opened up a new research area and has made it possible to identify human patterns that until now have not been understood and that have been improperly interpreted. This structure could certainly constitute a new ocular surface entity that could be included in the IVCM-ocular surface scoring system for future immunologic and toxicologic experiments and for the exploration of ocular surface disorders in pathologic conditions in humans.

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